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Report Title

Computer Simulations of the Virulome of Bacillus anthracis using Proteomics

ABSTRACT

Proteomic investigations of the biological warfare agent (BWA) *Bacillus anthracis* contributes to a comprehensive view of the cellular events that occur under host simulated conditions. The proteome of an organism is the sum of all proteins produced under defined conditions. It represents a global metabolic “snapshot” of a cell or microorganism at a particular moment. Presently there are large gaps in our knowledge about how *B. anthracis* infects and interacts with its host and how it causes its devastating consequences, what factors determine the host range, and the means by which *B. anthracis* invades host cells once it gains entry into the organism. Additionally, the contribution and interrelation of proteins encoded on each plasmid and the chromosome to the pathogenic process is currently unclear. To gain a detailed view of these virulent mechanisms results from investigations of various *B. anthracis* proteomes and subproteomes are used to create models of the pathogenic process. Furthermore the factors that determine host range as well as the dynamic changes associated with the release of secreted proteins (secretome) used by *B. anthracis* during invasion of the host were investigated. A time course analysis of the secretome and the identification of its key proteins were conducted using proteomics approaches. Subproteomic data of such global changes in the secretome were then used for computer simulations of the *B. anthracis* virulome. In addition, the subproteomes of *B. anthracis* that lack one of two plasmids (pXO1+, pXO2-); (pXO1-, pXO2+) or both plasmids (pXO1-, pXO2-) were compared. Using host reception labeled columns (fibronectin, heparin, etc) we also determined which *B. anthracis* proteins may be responsible in host cell invasion. Eluates of captured proteins were analyzed by LC-MS/MS to determine what pathogen proteins interact with specific receptors.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Number of Papers published in peer-reviewed journals: 0.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

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The Role of the *Bacillus anthracis* Secretome in Pathogenicity
Alexander Walz, Akbar Khan, and Vito DelVecchio
BioCOMP PI meeting in Arlington, VA; May 3-5 2005 (poster)

Bacillus anthracis Virulence Subproteomes
Vito DelVecchio, and Alexander Walz
BioCOMP PI meeting in Arlington, VA; May 3-5 2005 (oral presentation)

Dynamics of protein secretion in *Bacillus anthracis*
Alexander Walz, Akbar Khan, and Vito DelVecchio
BioCOMP PI meeting in Falls Church, VA; November 9-10, 2005 (poster)

Dynamics of protein secretion in *Bacillus anthracis*
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Total Number:	

Names of Post Doctorates

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FTE Equivalent:	
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Final Progress Report on:

**“Computer Simulations of the Virulome of *Bacillus anthracis*
using Proteomics”**

April 1st 2005 – July 31th 2006

From:	Vital Probes, Inc.
To:	Army Research Office, Research Triangle Park, NC
Contract No.:	W911NF-05-C-0047
Submitted by:	Vito G. DelVecchio, Ph.D.

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Final Report

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From: Vital Probes, Inc.
To: Army Research Office, Research Triangle Park, NC
Contract No.: W911NF-05-C-0047
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Statement of the problem studied

Proteomic investigations of the biological warfare agent (BWA) *Bacillus anthracis* contributes to a comprehensive view of the cellular events that occur under host simulated conditions. The proteome of an organism is the sum of all proteins produced under defined conditions. It represents a global metabolic “snapshot” of a cell or microorganism at a particular moment. Presently there are large gaps in our knowledge about how *B. anthracis* infects and interacts with its host and how it causes its devastating consequences, what factors determine the host range, and the means by which *B. anthracis* invades host cells once it gains entry into the organism. Additionally, the contribution and interrelation of proteins encoded on each plasmid and the chromosome to the pathogenic process is currently unclear. To gain a detailed view of these virulent mechanisms results from investigations of various *B. anthracis* proteomes and subproteomes are used to create models of the pathogenic process. Furthermore the factors that determine host range as well as the dynamic changes associated with the release of secreted proteins (secretome) used by *B. anthracis* during invasion of the host were investigated. A time course analysis of the secretome and the identification of its key proteins were conducted using proteomics approaches. Subproteomic data of such global changes in the secretome were then used for computer simulations of the *B. anthracis* virulome. In addition, the subproteomes of *B. anthracis* that lack one of two plasmids (pXO1⁺, pXO2⁻); (pXO1⁻, pXO2⁺) or both plasmids (pXO1⁻, pXO2⁻) were compared. Using host reception labeled columns (fibronectin, heparin, etc) we also determined which *B. anthracis* proteins may be responsible in host cell invasion. Eluates of captured proteins were analyzed by LC-MS/MS to determine what pathogen proteins interact with specific receptors.

Chromosome/plasmid cross-talk

The *Bacillus anthracis* genome consist of a 5.25 Mb chromosome and two large plasmids, pXO1 (182 kb) and pXO2 (95 kb). For example, three toxin genes are located on pXO1, while the capsule genes *cap* and *dep* are arranged in an operon on pXO2. The transcription of the toxin and capsule gene are influenced by host-related signals such as

temperature (37°C) and bicarbonate/CO₂. When *B. anthracis* is cultured at 37°C in a bicarbonate-containing minimal medium, toxin production is enhanced, with peak levels occurring at the end of exponential growth phase. To explore the relationship between plasmids and chromosome, a comparative proteomics study of the virulence attenuated toxinogenic, non-capsulated strain RA3R (pXO1⁺, pXO2⁻), the capsulated strain A3 (pXO1⁻, pXO2⁺), and the fully cured plasmid-less strain A74 (pXO1⁻, pXO2⁻) was performed. Proteins were isolated from cultures grown in R-medium containing glucose (0.25%, w/v) and sodium bicarbonate (0.85%, w/v) at 37°C under 5% CO₂, to simulate host conditions.

To elucidate the effect of different plasmid contents on the *B. anthracis* subproteomes membrane and cytosolic protein fractions were isolated from vegetative cell fractions using the Sigma PROTTWO Universal and PROTMEM Membrane Extraction Kit. These kits are designed to prepare highly enriched membrane protein and soluble/cytoplasmic protein fractions. Equal amounts of membrane and cytosolic proteins were trypsin digested and labeled with amine specific isobaric tags (iTRAQ), combined, and run on LC-MS/MS. The iTRAQ reagents allow multiplexed, amine-specific, stable isotope labeling of all peptides in up to four different samples simultaneously. The resulting MS/MS data were identified using the ProQuant software (Applied Biosystems). Table 1 below lists all LC-MS/MS detected membrane proteins in RA3R (pXO1⁺, pXO2⁻), A74 (pXO1⁻, pXO2⁻), and A3 (pXO1⁻, pXO2⁺). The presence or absence of a given protein is indicated by “+”= present and “-”= absent.

Table 2 lists all LC-MS/MS detected cytosolic proteins in *B. anthracis* RA3R (pXO1⁺, pXO2⁻), A74 (pXO1⁻, pXO2⁻), and A3 (pXO1⁻, pXO2⁺) grown under host simulated conditions. In addition table 2 lists cytosolic proteins that were isolated from RA3R cultures grown under non-induced (standard laboratory) conditions.

Examination of the contribution of each genetic element to production of proteins responsible for the pathogenic process and the characterization of new key regulators and pathways associated with this interplay help to predict the virulent activity of other pathogenic microorganisms as well as provide integral information for the development of simulation software.

Secretome experiments

Bacillus anthracis secretes numerous proteins into the host cells, extra cellular fluid, and tissues during infection. Strain RA3R (pXO1⁺/ pXO2⁻) was grown as described above in R-medium under induced conditions which simulate those encountered in the host. The secretome of the cells was harvested at 16h after inoculation and analyzed by LC-MS/MS analysis. A total of 274 extracellular proteins were identified by LC-MS/MS. All of the identified proteins were analyzed by SignalP, SecretomeP, PSORT, LipoP, TatP and TMHMM to characterize their predicted mode of secretion and cellular localization. Table 3 lists all secretome proteins identified by LC-MS/MS.

These secretome results have been included in the AnthraCyc web site at <http://biocyc.org/ANTHRA/server.html>. AnthraCyc is part of the BioCyc collection of pathway/genome databases which provides electronic reference sources on the pathways and genomes of more than 300 organisms. It provides a comprehensive collection of

experimentally elucidated metabolic pathways and reference data sets for computationally predicting metabolic pathways. It supports metabolic engineering, helps to compare biochemical networks, and serves as an encyclopedia of metabolism.

Secretome time course experiments

The *Bacillus anthracis* strain RA3R (pXO1⁺ pXO2⁻) extracellular proteome was analyzed at different phases of bacterial growth, *i.e.*, early, mid, and late log phase under conditions that simulate those encountered in the host. The secretome of the cells was harvested at 6h, 10h, and 16h time points after inoculation and analyzed by 2-D gel electrophoresis (Fig.1).

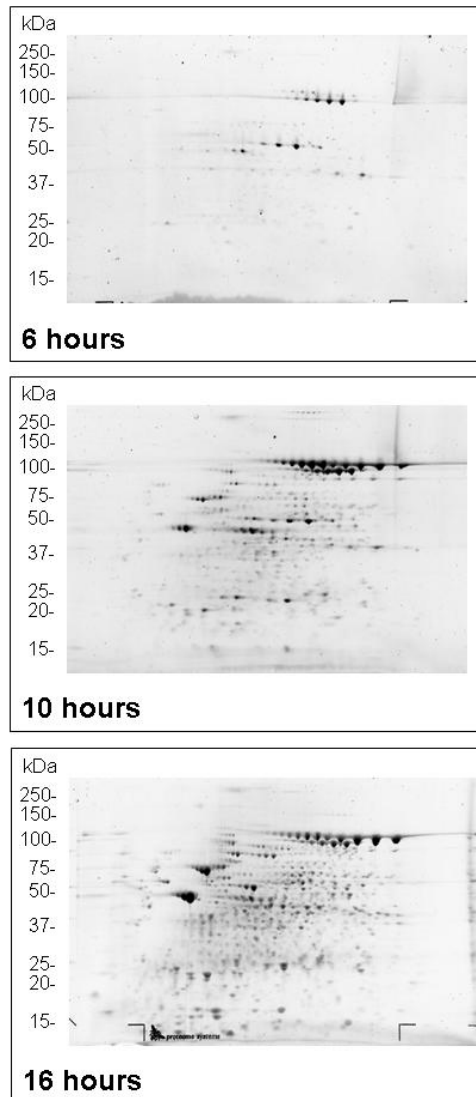


Fig. 1 2D Gel images of SYPRO[®] Ruby stained secretome proteins at 6, 10, and 16h time points. Each protein spot from one time point is compared to its counterparts using the Phoretix 2D Expression v2005 software (Nonlinear Dynamics).

Using Phoretix 2D Expression v2005 software average gels were generated. Four individual images of SYPRO Ruby stained 2D gels at each time point were used for averaging at each time point. The protein expression levels were deduced on the basis of “fold-difference” in averaged spot volume, which are derived from pixel intensity minus calculated background values. Gel image analysis reveal unique up- or down- regulated secretome proteins. Over- and underexpressed proteins are outlined in yellow and red, respectively (Fig. 2). A majority (> 99%) of extracellular proteins belong to the upregulated secretion. S-layer proteins Sap and EA1, PagA, and enolase were the most predominant proteins. To our knowledge, this is the first global quantitative analysis of *B. anthracis* protein secretion dynamics with respect to time following induction.

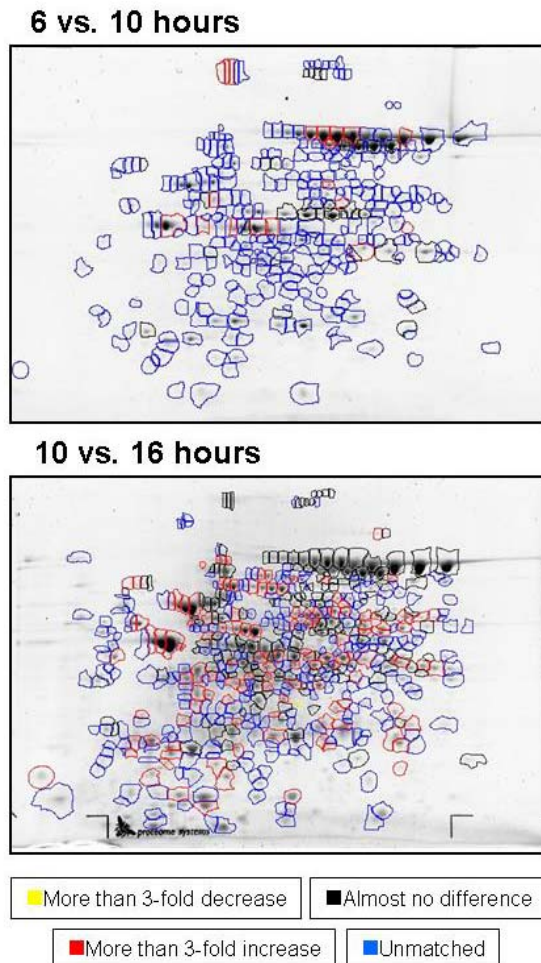


Fig. 2 Calculation of protein expression levels using Phoretix 2D Expression v2005 software

For protein annotation three replica gels of the 16h time point were made and all of the proteins were picked. Using Matrix Assisted Laser Desorption Ionization (MALDI) Time of Flight (TOF) Mass Spectrometry (MS) the protein identity and gi for the individual protein spots was identified. Table 4 lists the identified protein names and NCBI accession numbers (gi). The protein expression levels at the 16h time point were

used as reference for calculating the x-fold expression level difference at the 6h and 10h time point.

Certain proteins (i.e. protective antigen [PA]) are identified more than once. This may be due to the presence of protein isoforms that have charge and mass variations due to posttranslational modifications. Different isoforms are indicated as PA1, PA2, PA3, etc. These isoforms are identified by MALDI-TOF MS and expressed at different levels during the secretion process (Fig.3).

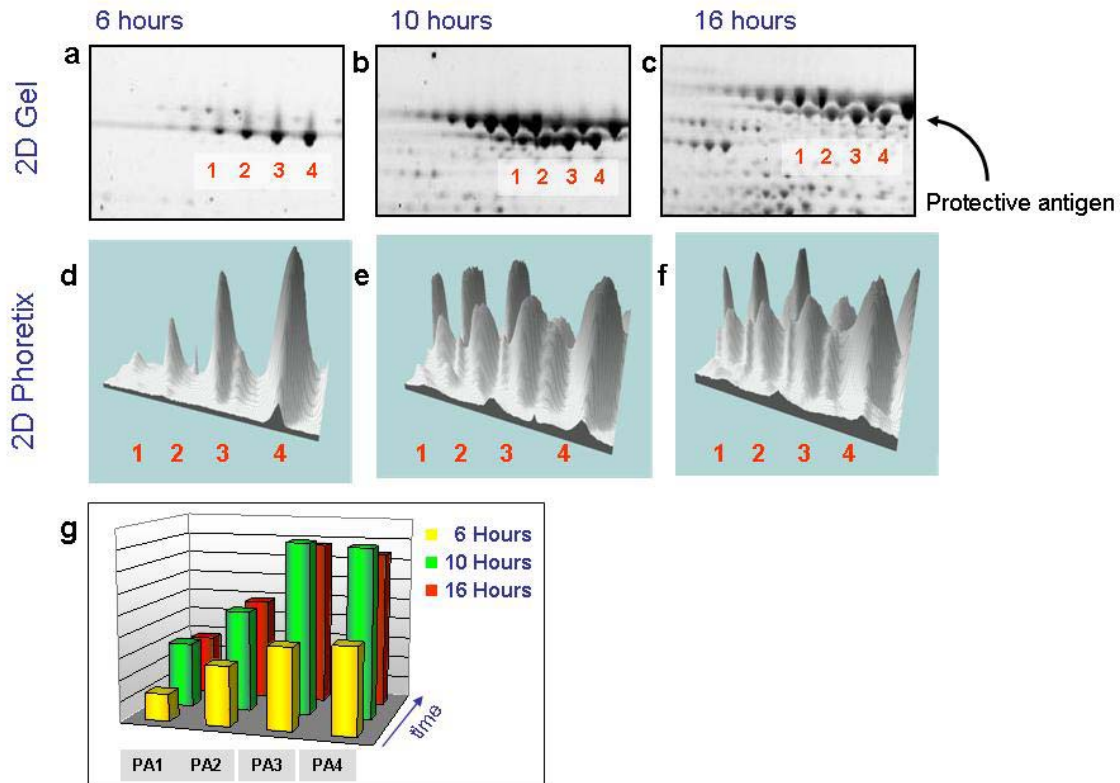


Fig. 3 Gel images (a-c) show SYPRO® Ruby stained PA at 6h, 10h, and 16h time points. Based on the Phoretix 2D analysis which calculates 3D images of the protein spots based on pixel intensity and size (e-f), absolute expression levels are calculated. Graph (g) shows the dynamics of protective antigen isoform expression over time.

Based on the Phoretix 2D analysis which calculates relative protein abundance using spot pixel intensity and size, relative expression levels were calculated from average gels. Four individual images of SYPRO Ruby stained 2D gels were used for averaging at each time point. For protein annotation protein spots from three replicate gels of the 16 h time point were identified using MALDI-TOF MS (Fig.4).

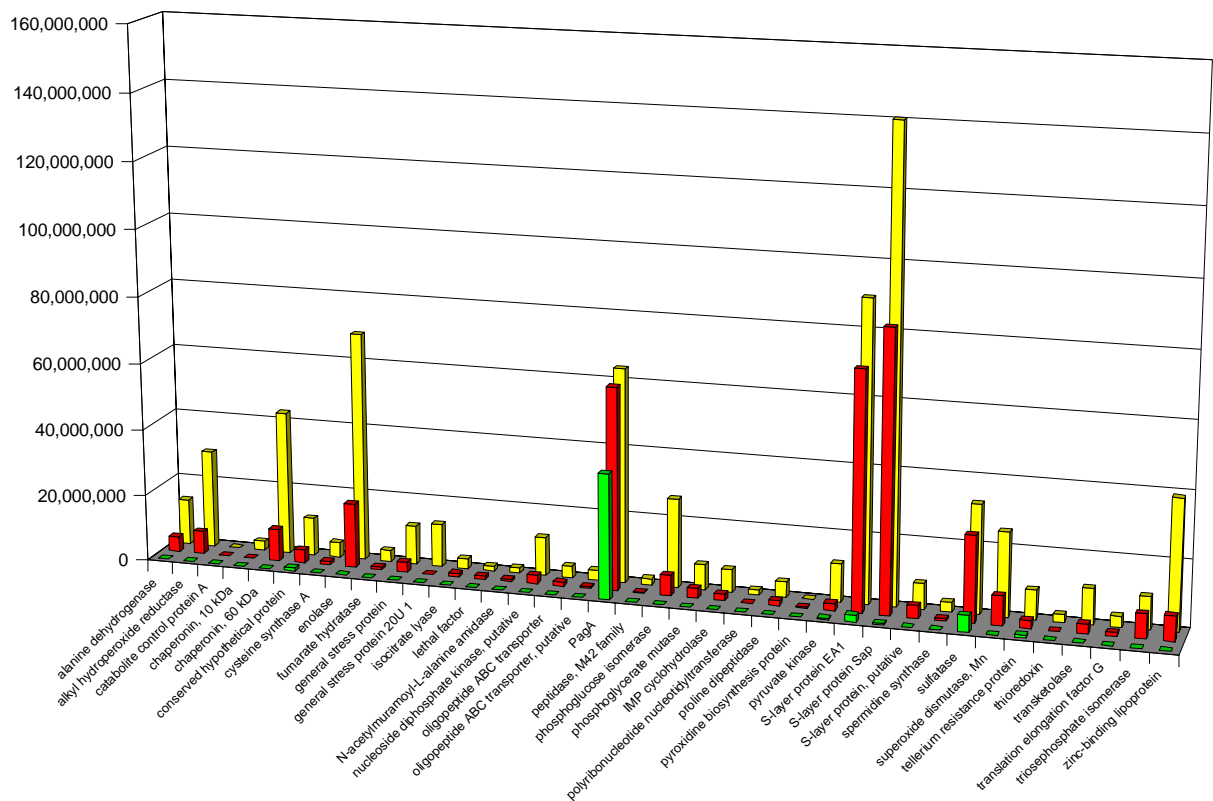


Fig. 4 Time point analysis of RA3R protein secretion based on Phoretix 2D results at 6h, 10h, and 16h.

Protein identification and quantification using iTRAQ and LC-MS/MS

In addition to 2D GE and MALDI-TOF we utilize LC-MS/MS and iTRAQ to determine differences in relative expression levels at different time points. The iTRAQ Reagents allow multiplexed, amine-specific, stable isotope labeling of all peptides in up to four different samples simultaneously. It also allows for relative quantification. Proteins from equal volumes of *B. anthracis* RA3R (pXO1⁺, pXO2⁻) secretome at different time points (6 hours, 10 hours and 16 hours) were first digested with trypsin and the subsequent tryptic peptides were labeled with different iTRAQ tags, combined and run on LC-MS/MS. The resulting MS/MS data were identified and quantified using ProQuant (Applied Biosystems). The iTRAQ tags are dissociated from the peptide during MS/MS and were used to calculate relative expression levels of peptide. The graph below shows the x-fold protein over- and underexpression relative to the 6h time point (Fig. 5).

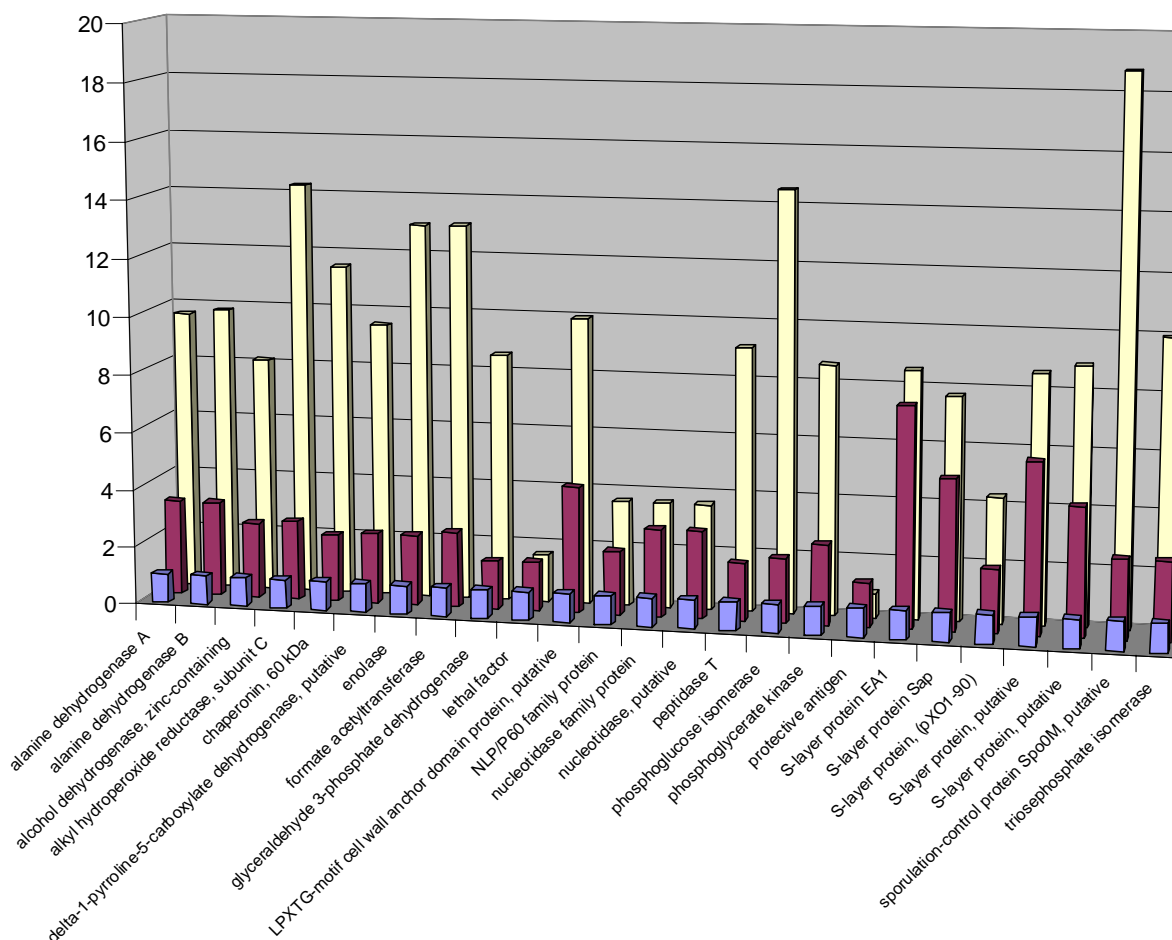


Fig. 5 Time point analysis of selected secretome proteins in *B. anthracis* RA3R using iTRAQ. The labeling allows the calculation of relative expression levels over 6h, 10h, and 16h.

The above results of *B. anthracis* protein secretion dynamics were incorporated in the BioCyc Pathway Tools Omics Viewer. This BioSpice tool allows the visualization of individual metabolic pathways, or to view the complete metabolic map of an organism. This database is based on nonredundant, experimentally elucidated metabolic pathways and contains pathways from more than 600 different organisms. The BioCyc Pathway Tools Omics Viewer can be used in a variety of scientific applications, such as providing a reference data set for computationally predicting the metabolic pathways of organisms from their sequenced genomes, supporting metabolic engineering, helping to compare biochemical networks, and serving as an encyclopedia of metabolism.

Our *B. anthracis* protein secretion data were used to create animated views of the time-course experiments. This enables the viewer to instantly see which pathways are active or inactive under certain sets of experimental conditions. Reaction steps in the metabolic overview are colored according to the corresponding data value. Similarly, compound nodes are colored according to the data value for the corresponding

compound. It also supplements the microarray data which can not describe if a gene is actually expressed or the gene product has a high turnover rate.

The animations can be accessed using the following links:

<http://www.ai.sri.com/~paley/anthra/absolute/anthra-absolute.html>

This figure shows the absolute expression levels of the various proteins, animated to show the three timepoints. The changes in expression levels for all proteins identified are based on 2DGE and MALDI-TOF results. Proteins with more than one isoform were grouped into one. Unlike the LC-MS/MS results that show relative increases in protein secretion for a given protein, the 2D/Maldi results allow the comparison of different expression levels of several proteins within a given time point.

<http://www.ai.sri.com/~paley/anthra/relative/anthra-relative.html>

This animation contains only two time points, 10h and 16h. The changes in protein over- and underexpression are represented relative to the 6h time point which was set to a value of one.

Moonlighting proteins

Several of the proteins that have been detected in the secretome have moonlighting functions. That means they are multifunctional proteins which have many different functions that are not only catalytic but structural or regulatory. Besides being secreted they are also located in the cytoplasm or membranes. There are actually many cases of proteins with more than one role in an organism. Table 5 compares the presence of all identified *B. anthracis* RA3R proteins in the different subproteomes. These new examples of moonlighting proteins add to our understanding of the potential importance of this group of proteins.

Evaluation of Chloroform to Inactivate *B. anthracis* Spores

To address the safety concerns of utilizing membrane and cytosolic protein fractions isolated from the virulent RA3 (pXO1⁺, pXO2⁺) strain the effect of chloroform on spores was investigated. One major concern when working with the virulent *B. anthracis* A5 strain is the possibility that some vegetative cells will sporulate during the growth process.

Non-pathogenic strains of *B. anthracis* were grown at VPI and subsequent killing of vegetative cells using chloroform treatment was evaluated. One-hundred microliters of a *B. anthracis* RA3R (pXO1⁺, pXO2⁻) spore suspension at 1.88×10^8 /ml in 1900µl of sterile molecular grade water were treated for 3, 4, 5, and 6h with chloroform with shaking. The treated cell fractions were then examined for viability and the presence of live spores. After 6h of incubation with chloroform a large percentage of *B. anthracis* spores germinated, thus preventing the use of the fully virulent A5 strain for proteomic membrane and cytosolic analysis.

Determine what *B. anthracis* proteins interact with host receptors

Bacillus anthracis RA3R secretome proteins that bind to the extracellular matrix binding proteins Fibronectin, Heparin, and Collagen were determined. *B. anthracis* RA3R (pXO1+/ pXO2-) was grown in R-medium under induced conditions. The secretome of the cells was harvested at 16h. Pierce MicroLink columns were labeled with the extracellular matrix binding proteins Fibronectin, Heparin, and Collagen. These columns allow for the covalent attachment of 25-100µg proteins through their primary amines/ lysine residues in a leach-resistant affinity support. The labeled columns were incubated with RA3R secretome proteins. Proteins that bind to these extracellular matrix proteins were identified using LC-MS/MS. These results of the binding protein experiments are important to determine which *B. anthracis* proteins may be responsible in host cell invasion. Table 6 lists all *Bacillus anthracis* RA3R secretome proteins that bind to the extracellular matrix binding proteins Fibronectin, Heparin, and Collagen.

Table 6 *B. anthracis* proteins interact with host receptors

Fibronectin:

gi|21392820| calmodulin-sensitive adenylate cyclase
gi|65317110| 3-phosphoglycerate kinase
gi|65317768| Peroxiredoxin
gi|65318302| Large exoproteins involved in heme utilization or adhesion
gi|30259273| conserved hypothetical protein
gi|30253764| chaperonin, 60 kDa

Heparin:

gi|65316889| Glucose-6-phosphate isomerase
gi|65317198| L-lactate permease
gi|30254056| glutamate synthase, large subunit, putative
gi|65321093| ABC-type multidrug transport system, ATPase component
gi|65321569| Biotin carboxylase
gi|30259273| conserved hypothetical protein

Collagen:

gi|10956354| hypothetical protein pxo1_107
gi|20520090| hypothetical protein, (pXO1-10)
gi|21392848| lethal factor
gi|65316889| Glucose-6-phosphate isomerase
gi|65317058| Tyrosyl-tRNA synthetase
gi|65317110| 3-phosphoglycerate kinase
gi|47505857| transposase, IS605 family
gi|65317161| Ribosome-associated protein Y (PSrp-1)
gi|65317202| Phosphoglycerol transferase and related proteins
gi|65317542| ATPases with chaperone activity, ATP-binding subunit
gi|47500581| ABC transporter, permease protein

gi|65317694| Co-chaperonin GroES (HSP10)
 gi|47500675| chaperonin, 60 kDa
 gi|65317767| Alkyl hydroperoxide reductase, large subunit
 gi|65317828| Uncharacterized proteins involved in stress response
 gi|65317829| Uncharacterized proteins involved in stress response
 gi|65317927| Pyruvate-formate lyase
 gi|65318302| Large exoproteins involved in heme utilization or adhesion
 gi|30254646| N-acetylmuramoyl-L-alanine amidase, family 3
 gi|30255392| branched-chain amino acid transport system II carrier protein
 gi|65318880| Nucleoside diphosphate kinase
 gi|30256915| hypothetical protein BA_2275
 gi|65319787| hypothetical protein Bant_01003124
 gi|30257604| conserved domain protein
 gi|30257881| S-layer protein, putative
 gi|65320784| Glycerophosphoryl diester phosphodiesterase
 gi|49182118| collagen adhesion protein, N-terminus
 gi|65320930| hypothetical protein Bant_01004371
 gi|30258263| transketolase
 gi|65321069| Carbamoylphosphate synthase large subunit
 gi|65321104| Predicted HD superfamily hydrolase
 gi|65321128| Aspartate-semialdehyde dehydrogenase
 gi|65321151| Ribosome recycling factor
 gi|65321390| hypothetical protein Bant_01004871
 gi|65321414| Acetyl-CoA acetyltransferase
 gi|30259005| superoxide dismutase, Mn
 gi|30259100| cystathionine beta-lyase
 gi|65321886| Thiol-disulfide isomerase and thioredoxins
 gi|65321966| 6-phosphofructokinase
 gi|30259568| hypothetical protein BA_5080
 gi|30254284| conserved hypothetical protein
 gi|30254854| isocitrate lyase
 gi|30255150| oligopeptide ABC transporter, oligopeptide-binding protein

Summary of the most important results

We have amassed significant proteomic data sets on the subproteomes of *B. anthracis* strains that differ in their plasmid content and generated an extensive overview of the secretion process. All of these datasets have been shared with the BioSPICE community and have been presented during the BioCOMP PI meeting in Arlington, VA May 3-5 2005, and Falls Church, VA November 9-10, 2005. This proteomics/ systems biology approach will help in our ability to simulate secretion of virulence factors for pathogens in general, independent of the source of infection. It will assist in the rapid identification of genetically engineered or new biological threat agents. This study also provided basic knowledge of *B. anthracis* which may be used in the development of improved detection technology, simulants, antimicrobial therapeutic measures and identification of new virulence factors and next generation vaccine candidate proteins.

Table 1. List of all LC-MS/MS detected membrane proteins in RA3R, A74, and A3

Accession #	Name	A74 (-/-) 114	RA3R (+/-) 115	A3 (-/+) 116
gi 47525336	negative regulator of genetic competence ClpC/MecB	+	+	+
gi 47525363	translation elongation factor G	+	+	+
gi 47525364	translation elongation factor Tu	+	+	+
gi 47525525	chaperonin, 10 kDa	+	+	+
gi 47525527	chaperonin, 60 kDa	+	+	+
gi 47525612	alkyl hydroperoxide reductase, subunit C	+	+	+
gi 47526172	S-layer protein Sap	+	+	+
gi 47526173	S-layer protein EA1	+	+	+
gi 47526261	S-layer protein, putative	+	+	+
gi 47526367	S-layer protein, putative	+	+	+
gi 47526398	S-layer protein, putative	+	+	+
gi 47526400	S-layer protein, putative	+	+	+
gi 47526596	3-oxoacyl-(acyl-carrier-protein) reductase, putative	+	+	+
gi 47526810	nucleoside diphosphate kinase, putative	+	+	+
gi 47527105	N-acetylmuramoyl-L-alanine amidase, family 3	+	+	+
gi 47527564	alcohol dehydrogenase, zinc-containing	+	+	+
gi 47529263	succinyl-CoA synthase, alpha subunit	+	+	+
gi 47529896	aldehyde-alcohol dehydrogenase	+	+	+
gi 47530139	phosphofructokinase	-	+	-
gi 47530168	universal stress protein family	+	+	+
gi 47530674	enolase	+	+	+
gi 47530679	glyceraldehyde 3-phosphate dehydrogenase	+	+	+
gi 47566444	S-layer protein, (pXO1-90)	-	+	-
gi 47566522	DNA topoisomerase I	-	+	-
gi 47778196	S-layer protein, putative	+	+	+
gi 47778326	conserved hypothetical protein	+	+	+
gi 47778335	isocitrate dehydrogenase, NADP-dependent	+	+	+
gi 47778387	iron compound ABC transporter, iron compound-binding protein	+	+	+

Table 2. List of all LC-MS/MS detected cytosolic proteins in RA3R, A74, A3, and uninduced RA3R cultures

Accession #	Name	A74 (-/-) induced	RA3R (+/-) induced	A3 (-/+) induced	RA3R (+/-) uninduced
gi 47525266	pyridoxine biosynthesis protein	+	+	+	+
gi 47525302	UDP-N-acetylglucosamine pyrophosphorylase	+	+	+	+
gi 47525321	cysteine synthase A	+	+	+	+
gi 47525336	negative regulator of genetic competence ClpC/MecB	+	+	+	+
gi 47525352	ribosomal protein L1	+	+	+	+
gi 47525355	ribosomal protein L7/L12	+	+	+	+
gi 47525363	translation elongation factor G	+	+	+	+
gi 47525364	translation elongation factor Tu	+	+	+	+
gi 47525369	ribosomal protein L2	+	+	+	+
gi 47525372	ribosomal protein S3	+	+	+	+
gi 47525378	ribosomal protein L5	+	+	+	+
gi 47525380	ribosomal protein S8	+	+	+	-
gi 47525383	ribosomal protein S5	+	+	+	+
gi 47525384	ribosomal protein L30	+	+	+	-
gi 47525525	chaperonin, 10 kDa	+	+	+	+
gi 47525527	chaperonin, 60 kDa	+	+	+	+
gi 47525554	phosphoribosylaminoimidazole-succinocarboxamide synthase	+	+	+	+
gi 47525561	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	+	+	+	+
gi 47525572	delta-1-pyrroline-5-carboxylate dehydrogenase, putative	+	+	+	+
gi 47525611	alkyl hydroperoxide reductase, F subunit	+	+	+	+
gi 47525645	conserved domain protein	+	+	+	+
gi 47525675	tellurite resistance protein, putative	+	+	+	+
gi 47525698	conserved hypothetical protein	+	+	+	+
gi 47525779	formate acetyltransferase	+	+	+	+
gi 47525869	alanine dehydrogenase	+	+	+	+
gi 47525997	phosphate ABC transporter, phosphate-binding protein, putative	+	-	-	-
gi 47526138	glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent	+	+	+	+
gi 47526172	S-layer protein Sap	+	+	+	+
gi 47526173	S-layer protein EA1	+	+	+	+
gi 47526347	ferrochelataase	+	+	+	+
gi 47526421	ornithine aminotransferase	+	+	+	+
gi 47526469	oligoendopeptidase F	+	+	+	+
gi 47526497	enoyl-(acyl-carrier-protein) reductase	+	+	+	+
gi 47526506	conserved hypothetical protein	+	+	+	+
gi 47526662	D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase	+	+	+	+
gi 47526805	DNA-binding protein HU	+	+	+	+
gi 47526810	nucleoside diphosphate kinase, putative	+	+	+	+

gi 47526862	thermostable carboxypeptidase 1	+	+	+	+
gi 47527091	response regulator	+	+	+	+
gi 47527105	N-acetylmuramoyl-L-alanine amidase, family 3	+	+	+	+
gi 47527328	NADH:flavin oxidoreductase / NADH oxidase family protein	+	+	+	+
gi 47527483	conserved hypothetical protein	+	+	+	+
gi 47527531	conserved hypothetical protein	+	+	+	+
gi 47527548	asparagine synthetase, glutamine-hydrolyzing	+	+	+	+
gi 47527564	alcohol dehydrogenase, zinc-containing	+	+	+	+
gi 47527607	sporulation-control protein Spo0M, putative	+	+	+	+
gi 47527649	methylmalonic acid semialdehyde dehydrogenase	+	+	+	+
gi 47527664	isochorismate synthase Dhbc	+	+	+	+
gi 47527800	methylmalonic acid semialdehyde dehydrogenase	+	+	+	+
gi 47527903	D-alanine--D-alanine ligase	+	+	+	+
gi 47527984	conserved hypothetical protein	+	+	+	+
gi 47528056	transcriptional regulator, DeoR family	+	+	+	-
gi 47528252	chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase	+	+	+	+
gi 47528623	S-layer protein, putative	+	+	+	+
gi 47528716	transaldolase, putative	+	+	+	+
gi 47528837	oligoendopeptidase F	+	+	+	+
gi 47528927	oligopeptide ABC transporter, oligopeptide-binding protein, putative	+	+	+	+
gi 47528928	oligopeptide ABC transporter, oligopeptide-binding protein, putative	+	+	+	+
gi 47528961	aconitate hydratase 1	+	+	+	+
gi 47529031	conserved hypothetical protein UPF0154	+	+	+	+
gi 47529201	pyruvate ferredoxin oxidoreductase, alpha subunit, putative	+	+	+	+
gi 47529208	competence/damage-inducible protein CinA	+	+	-	-
gi 47529220	lipoprotein, Bmp family	+	+	+	+
gi 47529227	aspartate kinase, monofunctional class	+	+	+	+
gi 47529234	polyribonucleotide nucleotidyltransferase	+	-	+	-
gi 47529254	translation elongation factor Ts	+	+	+	+
gi 47529263	succinyl-CoA synthase, alpha subunit	+	+	+	+
gi 47529264	succinyl-CoA synthase, beta subunit	+	+	+	+
gi 47529279	acyl carrier protein	+	+	+	+
gi 47529328	cell-division initiation protein DivIVA	+	+	+	+
gi 47529479	pyruvate dehydrogenase complex E1 component, beta subunit	+	+	+	+
gi 47529511	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	+	+	+	+
gi 47529560	phosphoenolpyruvate-protein phosphotransferase	+	+	+	+
gi 47529575	peptidyl-prolyl cis-trans isomerase, cyclophilin-type	+	+	+	+
gi 47529678	3-methyl-2-oxobutanoate dehydrogenase, beta subunit	+	+	+	+
gi 47529682	leucine dehydrogenase	+	+	+	+
gi 47529742	glycine cleavage system P protein, subunit 2	+	+	+	+
gi 47529744	glycine cleavage system T protein	+	+	+	+
gi 47529836	chaperone protein dnaK	+	+	+	+

gi 47529881	phenylalanine-4-hydroxylase, putative	+	+	+	+
gi 47529896	aldehyde-alcohol dehydrogenase	+	+	+	+
gi 47529897	cystathionine beta-lyase	+	+	+	+
gi 47530004	trigger factor	+	+	+	+
gi 47530057	thioredoxin	+	+	+	+
gi 47530058	electron transfer flavoprotein, alpha subunit	+	+	+	+
gi 47530065	iron compound ABC transporter, iron compound-binding protein	+	+	+	+
gi 47530113	conserved hypothetical protein	+	+	+	+
gi 47530114	ribosomal protein L20	+	+	+	+
gi 47530115	ribosomal protein L35	+	+	+	+
gi 47530138	pyruvate kinase	+	+	+	+
gi 47530139	phosphofructokinase	+	+	+	+
gi 47530143	malate dehydrogenase, putative	+	+	+	+
gi 47530155	proline dipeptidase	+	+	+	+
gi 47530168	universal stress protein family	+	+	+	+
gi 47530203	ribosomal protein S4	+	+	+	+
gi 47530411	naphthoate synthase	+	+	+	+
gi 47530435	phosphoglucose isomerase	+	+	+	+
gi 47530451	glycyl-tRNA synthetase	+	+	+	+
gi 47530456	UTP-glucose-1-phosphate uridylyltransferase	+	+	+	+
gi 47530465	pyridine nucleotide-disulphide oxidoreductase	+	+	+	+
gi 47530507	transcriptional activator tipA, putative	+	+	+	+
gi 47530523	ABC transporter, ATP-binding protein	+	+	+	+
gi 47530544	L-lactate dehydrogenase	+	+	+	+
gi 47530616	conserved domain protein	+	+	+	+
gi 47530622	pyridine nucleotide-disulphide oxidoreductase	+	+	+	+
gi 47530674	enolase	+	+	+	+
gi 47530678	phosphoglycerate kinase	+	+	+	+
gi 47530679	glyceraldehyde 3-phosphate dehydrogenase	+	+	+	+
gi 47530734	preprotein translocase, SecA subunit	+	+	+	+
gi 47530867	ATP synthase F1, beta subunit	+	+	+	+
gi 47530869	ATP synthase F1, alpha subunit	+	+	+	+
gi 47530870	ATP synthase F1, delta subunit	+	+	+	+
gi 47530893	ribosomal protein L31	+	+	+	+
gi 47530963	conserved hypothetical protein	+	+	+	+
gi 47531052	DNA-binding response regulator YycF	+	+	+	+
gi 47531059	single-stranded DNA-binding protein	+	+	+	+
gi 47566503	conserved hypothetical protein, (pXO1-97)	-	+	-	+
gi 47566506	zinc-binding lipoprotein AdcA domain protein, (pXO1-130)	-	+	-	+
gi 47566508	conserved hypothetical protein, (pXO1-131)	-	+	-	+
gi 47777774	dihydroneopterin aldolase	+	+	+	+
gi 47777783	conserved hypothetical protein	+	+	+	+

gi 47777891	uroporphyrinogen decarboxylase	-	+	-	-
gi 47778274	integrase/recombinase XerD	+	+	+	+
gi 47778276	acetyltransferase, GNAT family	-	+	+	+
gi 47778313	conserved hypothetical protein	+	+	+	+
gi 47778335	isocitrate dehydrogenase, NADP-dependent	+	+	+	+
gi 47778387	iron compound ABC transporter, iron compound-binding protein	+	+	+	+
gi 47778388	ribonuclease R	+	+	+	+
gi 47778395	triosephosphate isomerase	+	+	+	+
gi 50196909	ribosomal protein L16	+	+	+	+

Table 3. List of all LC-MS/MS detected secretome proteins

Accession #	Name
gi 47525264	inosine-5'-monophosphate dehydrogenase
gi 47525266	pyridoxine biosynthesis protein
gi 47525268	seryl-tRNA synthetase
gi 47525302	UDP-N-acetylglucosamine pyrophosphorylase
gi 47525317	hypoxanthine phosphoribosyltransferase
gi 47525321	cysteine synthase A
gi 47525336	negative regulator of genetic competence ClpC/MecB
gi 47525352	ribosomal protein L1
gi 47525354	ribosomal protein L10
gi 47525355	ribosomal protein L7/L12
gi 47525355	ribosomal protein L7/L12
gi 47525359	DNA-directed RNA polymerase, beta' subunit
gi 47525362	ribosomal protein S7
gi 47525363	translation elongation factor G
gi 47525364	translation elongation factor Tu
gi 47525378	ribosomal protein L5
gi 47525381	ribosomal protein L6
gi 47525383	ribosomal protein S5
gi 47525387	adenylate kinase
gi 47525393	DNA-directed RNA polymerase, alpha subunit
gi 47525415	phosphoglucosamine mutase
gi 47525416	glucosamine--fructose-6-phosphate aminotransferase (isomerizing)
gi 47525525	chaperonin, 10 kDa
gi 47525527	chaperonin, 60 kDa
gi 47525553	adenylosuccinate lyase
gi 47525561	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
gi 47525562	phosphoribosylamine--glycine ligase
gi 47525572	delta-1-pyrroline-5-carboxylate dehydrogenase, putative
gi 47525588	glutamyl-tRNA(Gln) amidotransferase, B subunit
gi 47525611	alkyl hydroperoxide reductase, F subunit
gi 47525612	alkyl hydroperoxide reductase, subunit C
gi 47525665	type I phosphodiesterase/nucleotide pyrophosphatase family protein
gi 47525672	tellurium resistance protein
gi 47525779	formate acetyltransferase
gi 47525801	glutamate-1-semialdehyde-2,1-aminomutase
gi 47525869	alanine dehydrogenase
gi 47525869	alanine dehydrogenase
gi 47525874	nicotinate phosphoribosyltransferase, putative
gi 47525898	8-amino-7-oxononanoate synthase, putative
gi 47525951	transaldolase, putative
gi 47525965	conserved hypothetical protein
gi 47526016	thiazole biosynthesis protein ThiG
gi 47526082	conserved hypothetical protein
gi 47526085	conserved hypothetical protein
gi 47526115	conserved hypothetical protein
gi 47526138	glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent
gi 47526151	conserved hypothetical protein
gi 47526172	S-layer protein Sap
gi 47526173	S-layer protein EA1
gi 47526261	S-layer protein, putative
gi 47526292	transcriptional regulator, TetR family
gi 47526319	protein export protein prsA
gi 47526321	protease production regulatory protein Hpr
gi 47526347	ferrochelatase
gi 47526367	S-layer protein, putative
gi 47526399	S-layer protein, putative
gi 47526400	S-layer protein, putative
gi 47526401	malate synthase A
gi 47526402	isocitrate lyase
gi 47526404	cold shock protein CspA
gi 47526421	ornithine aminotransferase
gi 47526431	conserved hypothetical protein
gi 47526461	oligopeptide ABC transporter, oligopeptide-binding protein
gi 47526469	oligoendopeptidase F
gi 47526497	enoyl-(acyl-carrier-protein) reductase
gi 47526506	conserved hypothetical protein
gi 47526546	conserved hypothetical protein
gi 47526566	spermidine/putrescine ABC transporter, spermidine/putrescine-binding protein
gi 47526625	oligoendopeptidase F, putative
gi 47526645	ribonucleoside-diphosphate reductase, beta subunit
gi 47526662	D-alanine-activating enzyme/D-alanine-D-alanyl carrier protein ligase
gi 47526666	flavodoxin
gi 47526703	imidazoleglycerol phosphate synthase, cyclase subunit

gi 47526711	sulfatase
gi 47526757	purine nucleoside phosphorylase
gi 47526771	DNA-binding response regulator ResD
gi 47526800	glycerol-3-phosphate dehydrogenase (NAD(P)+)
gi 47526805	DNA-binding protein HU
gi 47526810	nucleoside diphosphate kinase, putative
gi 47526813	histidinol-phosphate aminotransferase
gi 47526862	thermostable carboxypeptidase 1
gi 47526874	rhodanese-like domain protein
gi 47526914	cold shock protein CspB
gi 47526990	hypothetical protein GBAA1701
gi 47526993	conserved hypothetical protein
gi 47527059	fumarate hydratase, class II
gi 47527105	N-acetylmuramoyl-L-alanine amidase, family 3
gi 47527222	S-layer protein, putative
gi 47527269	lipoprotein, putative
gi 47527280	hypothetical protein GBAA1985
gi 47527281	conserved hypothetical protein
gi 47527295	NH(3)-dependent NAD(+) synthetase
gi 47527311	general stress protein
gi 47527353	CBS domain protein
gi 47527411	metallo-beta-lactamase family protein
gi 47527483	conserved hypothetical protein
gi 47527526	conserved hypothetical protein
gi 47527529	conserved hypothetical protein
gi 47527531	conserved hypothetical protein
gi 47527534	penicillin-binding protein 3
gi 47527536	conserved hypothetical protein
gi 47527548	asparagine synthetase, glutamine-hydrolyzing
gi 47527564	alcohol dehydrogenase, zinc-containing
gi 47527607	sporulation-control protein Spo0M, putative
gi 47527664	isochorismate synthase DhbC
gi 47527666	isochorismatase
gi 47527715	cold shock protein CspA
gi 47527720	hydrolase, haloacid dehalogenase-like family
gi 47527787	hypothetical protein GBAA2500
gi 47527829	conserved domain protein
gi 47527903	D-alanine--D-alanine ligase
gi 47528004	conserved hypothetical protein
gi 47528119	inorganic pyrophosphatase, manganese-dependent
gi 47528239	sulfatase
gi 47528252	chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase
gi 47528392	Signal peptidase I U
gi 47528454	5'-nucleotidase, putative
gi 47528623	S-layer protein, putative
gi 47528814	hypothetical protein GBAA3529
gi 47528844	glycerophosphoryl diester phosphodiesterase, putative
gi 47528880	cold shock protein CspB
gi 47528895	aldehyde dehydrogenase
gi 47528945	serine protease
gi 47528961	aconitase hydratase 1
gi 47528968	alanyl-tRNA synthetase family protein
gi 47529026	N-acetylmuramoyl-L-alanine amidase, family 2
gi 47529034	transketolase
gi 47529035	transposase, IS605 family
gi 47529118	glutamine synthetase, type I
gi 47529132	conserved domain protein
gi 47529165	hypothetical protein GBAA3874
gi 47529183	sulfatase
gi 47529193	DNA mismatch repair protein MutL
gi 47529203	stage V sporulation protein S
gi 47529215	conserved hypothetical protein
gi 47529220	lipoprotein, Bmp family
gi 47529228	aspartate-semialdehyde dehydrogenase
gi 47529234	polyribonucleotide nucleotidyltransferase
gi 47529240	translation initiation factor IF-2
gi 47529252	ribosome recycling factor
gi 47529254	translation elongation factor Ts
gi 47529255	ribosomal protein S2
gi 47529256	transcriptional regulator CodY
gi 47529257	ATP-dependent hsl protease, ATP-binding subunit hslU
gi 47529263	succinyl-CoA synthase, alpha subunit
gi 47529264	succinyl-CoA synthase, beta subunit
gi 47529279	acyl carrier protein
gi 47529280	3-oxoacyl-(acyl-carrier-protein) reductase
gi 47529281	malonyl CoA-acyl carrier protein transacylase
gi 47529319	carbamoyl-phosphate synthase, small subunit

gi 47529327	isoleucyl-tRNA synthetase
gi 47529328	cell-division initiation protein DivIVA
gi 47529345	phospho-N-acetylmuramoyl-pentapeptide-transferase
gi 47529446	conserved hypothetical protein
gi 47529479	pyruvate dehydrogenase complex E1 component, beta subunit
gi 47529480	pyruvate dehydrogenase complex E1 component, alpha subunit
gi 47529489	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase, putative
gi 47529504	lipoprotein, putative
gi 47529522	maltosaccharide ABC transporter, maltosaccharide-binding protein, putative
gi 47529533	acetyl-CoA acetyltransferase
gi 47529560	phosphoenolpyruvate-protein phosphotransferase
gi 47529561	phosphocarrier protein HPr
gi 47529575	peptidyl-prolyl cis-trans isomerase, cyclophilin-type
gi 47529620	5'-nucleotidase family protein
gi 47529638	adenosylmethionine--8-amino-7-oxononanoate aminotransferase
gi 47529642	2',3'-cyclic-nucleotide 2'-phosphodiesterase
gi 47529652	hypothetical protein GBAA4356
gi 47529663	peptidase T
gi 47529678	3-methyl-2-oxobutanoate dehydrogenase, beta subunit
gi 47529682	leucine dehydrogenase
gi 47529699	methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase
gi 47529715	proline dipeptidase
gi 47529742	glycine cleavage system P protein, subunit 2
gi 47529743	glycine cleavage system P-protein, subunit 1
gi 47529744	glycine cleavage system T protein
gi 47529761	conserved hypothetical protein
gi 47529782	glucokinase
gi 47529794	superoxide dismutase, Mn
gi 47529803	endonuclease IV
gi 47529836	chaperone protein dnaK
gi 47529896	aldehyde-alcohol dehydrogenase
gi 47529897	cystathionine beta-lyase
gi 47529897	cystathionine beta-lyase
gi 47529924	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase
gi 47529926	rrf2 family protein
gi 47529931	histidyl-tRNA synthetase
gi 47529989	valyl-tRNA synthetase
gi 47530004	trigger factor
gi 47530057	thioredoxin
gi 47530058	electron transfer flavoprotein, alpha subunit
gi 47530059	electron transfer flavoprotein, beta subunit
gi 47530060	enoyl-CoA hydratase/isomerase family protein
gi 47530065	iron compound ABC transporter, iron compound-binding protein
gi 47530086	conserved domain protein
gi 47530087	LPXTG-motif cell wall anchor domain protein, putative
gi 47530098	asparaginyl-tRNA synthetase
gi 47530099	phenylalanyl-tRNA synthetase, beta subunit
gi 47530114	ribosomal protein L20
gi 47530115	ribosomal protein L35
gi 47530122	S-adenosylmethionine decarboxylase proenzyme
gi 47530132	malate dehydrogenase
gi 47530134	citrate synthase CitZ
gi 47530138	pyruvate kinase
gi 47530139	phosphofructokinase
gi 47530155	proline dipeptidase
gi 47530166	alanine dehydrogenase
gi 47530168	universal stress protein family
gi 47530183	acetate kinase
gi 47530223	catabolite control protein A
gi 47530346	autoinducer-2 production protein LuxS
gi 47530411	naphthoate synthase
gi 47530419	cold shock protein CspD
gi 47530429	L-lactate dehydrogenase
gi 47530435	phosphoglucose isomerase
gi 47530451	glycyl-tRNA synthetase
gi 47530456	UTP-glucose-1-phosphate uridylyltransferase
gi 47530465	pyridine nucleotide-disulphide oxidoreductase
gi 47530492	nifU domain protein
gi 47530499	phosphatase,haloacid dehalogenase family
gi 47530521	aminotransferase, class V
gi 47530523	ABC transporter, ATP-binding protein
gi 47530544	L-lactate dehydrogenase
gi 47530551	acyl-CoA dehydrogenase
gi 47530597	general stress protein 20U
gi 47530665	prophage LambdaBa03, terminase, large subunit, putative
gi 47530674	enolase
gi 47530675	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent

gi 47530678	phosphoglycerate kinase
gi 47530679	glyceraldehyde 3-phosphate dehydrogenase
gi 47530736	ribosomal subunit interface protein
gi 47530784	sulfatase
gi 47530798	conserved domain protein
gi 47530867	ATP synthase F1, beta subunit
gi 47530869	ATP synthase F1, alpha subunit
gi 47530877	uracil phosphoribosyltransferase
gi 47530878	serine hydroxymethyltransferase
gi 47530901	fructose-bisphosphate aldolase, class II
gi 47530963	conserved hypothetical protein
gi 47531052	DNA-binding response regulator YycF
gi 47531053	adenylosuccinate synthetase
gi 47531060	ribosomal protein S6
gi 47566376	IS231-related, transposase, (pXO1-36)
gi 47566444	S-layer protein, (pXO1-90)
gi 47566448	UTP-glucose-1-phosphate uridylyltransferase, (pXO1-94)
gi 47566456	calmodulin-sensitive adenylate cyclase
gi 47566476	protective antigen
gi 47566484	lethal factor
gi 47566506	zinc-binding lipoprotein AdcA domain protein, (pXO1-130)
gi 47566514	response regulator, putative
gi 47566700	hypothetical protein, (pXO2-29/30)
gi 47777767	methionyl-tRNA synthetase
gi 47777770	stage V sporulation protein G
gi 47777776	ribosomal protein L11
gi 47777798	GMP synthase
gi 47777980	L-lactate dehydrogenase
gi 47777984	NLP/P60 family protein
gi 47778144	conserved hypothetical protein
gi 47778178	lipoprotein, putative
gi 47778181	flavodoxin
gi 47778196	S-layer protein, putative
gi 47778274	integrase/recombinase XerD
gi 47778323	conserved hypothetical protein
gi 47778326	conserved hypothetical protein
gi 47778335	isocitrate dehydrogenase, NADP-dependent
gi 47778339	thiol peroxidase
gi 47778387	iron compound ABC transporter, iron compound-binding protein
gi 47778395	triosephosphate isomerase
gi 47778401	cold shock protein CspC
gi 47778402	endopeptidase IytE, putative
gi 47778412	fructose-1,6-bisphosphatase, class II
gi 50196909	ribosomal protein L16
gi 50196922	oligopeptide ABC transporter, oligopeptide-binding protein
gi 50196926	ribonucleoside-diphosphate reductase, alpha subunit, group I intron-containing
gi 50196966	conserved hypothetical protein TIGR00730

Table 4. List of B. anthracis proteins that show changes in expression levels at 6h, 10h, and 16hrs, relative to the 16h time point

Spot #	Protein	Accession No.	Expression Level compared to 16hrs 6hrs	Expression Level compared to 16hrs 10hrs	Expression Level at 16hrs *
1	oligopeptide ABC transporter, oligopeptide-binding protein 1	gi 30261296 ref NP_843673.	-	-4.222	2,136,052.92
2	alanine dehydrogenase	gi 30264697 ref NP_847074.	-	-2.933	6,788,711.29
3	alanine dehydrogenase 1	gi 30264697 ref NP_847074.	-	-3.128	6,758,631.48
4	alkyl hydroperoxide reductase, subunit C	gi 30260515 ref NP_842892.	-	-3.344	2,935,427.59
5	alkyl hydroperoxide reductase, subunit C 1	gi 30260515 ref NP_842892.	-	-2.972	10,518,930.79
6	alkyl hydroperoxide reductase, subunit C 2	gi 30260515 ref NP_842892.	-	-4.330	4,618,028.40
7	alkyl hydroperoxide reductase, subunit C 3	gi 30260515 ref NP_842892.	-	-14.223	6,052,868.08
8	alkyl hydroperoxide reductase, subunit C 4	gi 30260515 ref NP_842892.	-	-	3,108,336.38
9	alkyl hydroperoxide reductase, subunit C 5	gi 30260515 ref NP_842892.	-	-5.486	1,814,168.76
10	catabolite control protein A	gi 30264750 ref NP_847127.	-	-	23,316.70
11	chaperonin, 10 kDa	gi 30260442 ref NP_842819.	-	-	2,744,702.49
12	chaperonin, 60 kDa	gi 30260443 ref NP_842820.	-	-7.153	2,314,581.83
13	chaperonin, 60 kDa 1	gi 30260443 ref NP_842820.	-	-5.846	5,188,984.44
14	chaperonin, 60 kDa 2	gi 30260443 ref NP_842820.	-	-4.754	11,387,133.75
15	chaperonin, 60 kDa 3	gi 30260443 ref NP_842820.	-	-4.043	24,032,813.71
16	conserved hypothetical protein	gi 30262249 ref NP_844626.	-5.967	-2.351	5,184,790.25
17	conserved hypothetical protein 1	gi 30262249 ref NP_844626.	-	-3.965	3,957,886.71
18	conserved hypothetical protein 2	gi 30262249 ref NP_844626.	-	-	1,681,979.64
19	conserved hypothetical protein 3	gi 30265410 ref NP_847787.	-	-1.742	426,295.69
20	cysteine synthase A	gi 30260259 ref NP_842636.	-	-7.184	3,382,230.81
21	cysteine synthase A 1	gi 30260259 ref NP_842636.	-	-1.709	955,419.26
22	enolase	gi 30265161 ref NP_847538.	-244.688	-3.343	44,176,362.71
23	enolase 1	gi 30265161 ref NP_847538.	-	-3.856	18,184,768.19
24	enolase 2	gi 30265161 ref NP_847538.	-	-4.122	6,180,508.28
25	fumarate hydratase, class II	gi 30261818 ref NP_844195.	-	-4.110	3,440,373.90
26	general stress protein	gi 30262040 ref NP_844417.	-	-3.052	9,079,087.24
27	general stress protein 1	gi 30262040 ref NP_844417.	-	-	2,446,362.38
28	general stress protein 20U 1	gi 30265092 ref NP_847469.	-	-	12,809,765.71
29	isocitrate lyase	gi 30261240 ref NP_843617.	-	-2.936	3,023,269.55
30	lethal factor	gi 47566484 ref YP_016503.2	-	-1.634	810,857.72
31	lethal factor 1	gi 47566484 ref YP_016503.2	-	-1.843	564,826.05
32	Mixture from proteins:"gi 30263804 ref NP_846181.1";"gi 30264442 ref NP_846819.1"	Mixture from proteins	-	-5.546	2,563,889.49
33	Mixture from proteins:"gi 30263804 ref NP_846181.1";"gi 30264442 ref NP_846819.1"	Mixture from proteins	-	-4.893	5,722,103.48
34	Mixture from proteins:"gi 47566476 ref YP_016495.2";"gi 47566484 ref YP_016503.2"	Mixture from proteins	-1.806	1.087	14,800,901.76
35	Mixture from proteins:"gi 47566484 ref YP_016503.2";"gi 47566476 ref YP_016495.2"	Mixture from proteins	-	-1.503	1,931,716.79
36	Mixture from proteins:"gi 47566484 ref YP_016503.2";"gi 47566476 ref YP_016495.2"	Mixture from proteins	-2.173	1.096	5,630,211.08
37	N-acetylmuramoyl-L-alanine amidase, family 2	gi 30263621 ref NP_845998.	-	-2.516	1,675,760.16
38	nucleoside diphosphate kinase, putative	gi 30261610 ref NP_843987.	-	-	3,372,807.93
39	nucleoside diphosphate kinase, putative 1	gi 30261610 ref NP_843987.	-	-3.284	8,259,629.97
40	oligopeptide ABC transporter, oligopeptide-binding protein	gi 30261296 ref NP_843673.	-	-2.142	1,501,943.70
41	PagA	gi 47566476 ref YP_016495.2	-1.520	1.019	2,683,047.49
42	PagA 1	gi 47566476 ref YP_016495.2	1.053	1.194	5,246,913.27
43	PagA 2	gi 47566476 ref YP_016495.2	1.257	1.220	7,394,368.07
44	PagA 5	gi 47566476 ref YP_016495.2	-2.002	1.054	15,546,894.54
45	PagA 8	gi 47566476 ref YP_016495.2	-	-2.515	945,906.02
46	PagA 9	gi 47566476 ref YP_016495.2	-	-1.197	1,121,234.63

* Expression level values are based on spot volume and intensity as calculated by Phoretix 2D software

47	PagA 10	gi 47566476 ref YP_016495.2	-	-1.815	1,455,206.70
48	PagA 14	gi 47566476 ref YP_016495.2	-3.310	-1.053	3,300,303.12
49	PagA 15	gi 47566476 ref YP_016495.2	-1.720	-1.004	9,671,456.38
50	PagA 16	gi 47566476 ref YP_016495.2	1.222	1.106	1,755,199.11
51	PagA 17	gi 47566476 ref YP_016495.2	-2.319	-1.259	3,236,443.59
52	PagA 18	gi 47566476 ref YP_016495.2	-2.001	-1.034	4,591,395.38
53	PagA 19	gi 47566476 ref YP_016495.2	-	-	4,732,934.22
54	PagA 20	gi 47566476 ref YP_016495.2	-4.514	1.010	2,373,332.38
55	peptidase, M42 family	gi 30264641 ref NP_847018.	-	-6.389	1,868,069.60
56	phosphoglucose isomerase	gi 30264939 ref NP_847316.	-	-3.905	14,866,884.98
57	phosphoglucose isomerase 1	gi 30264939 ref NP_847316.	-	-4.379	7,836,909.71
58	phosphoglucose isomerase 3	gi 30264939 ref NP_847316.	-	-6.822	3,817,583.58
59	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	gi 30265162 ref NP_847539.	-	-2.744	1,567,427.92
60	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	gi 30265162 ref NP_847539.	-	-2.424	6,060,000.93
61	phosphoribosylaminimidazolecarboxamide formyltransferase/IMP cyclohydrolase	gi 30260471 ref NP_842848.	-	-3.064	3,948,887.13
62	phosphoribosylaminimidazolecarboxamide formyltransferase/IMP cyclohydrolase 1	gi 30260471 ref NP_842848.	-	-3.993	2,870,118.45
63	polysulfonucleotide nucleotidyltransferase	gi 30263811 ref NP_846188.	-	-	931,160.08
64	polysulfonucleotide nucleotidyltransferase 1	gi 30263811 ref NP_846188.	-	-	452,215.53
65	proline dipeptidase	gi 30264273 ref NP_846650.	-	-2.861	3,297,747.14
66	proline dipeptidase 1	gi 30264273 ref NP_846650.	-	-3.038	1,267,916.33
67	pyroxidine biosynthesis protein	gi 30260204 ref NP_842581.	-	3.733	103,874.09
68	pyruvate kinase	gi 30264669 ref NP_847046.	-6.591	-6.565	2,316,350.59
69	pyruvate kinase 1	gi 30264669 ref NP_847046.	-	-10.812	1,282,504.13
70	pyruvate kinase 2	gi 30264669 ref NP_847046.	-	-6.909	1,378,564.60
71	pyruvate kinase 3	gi 30264669 ref NP_847046.	-	-5.724	2,116,676.86
72	pyruvate kinase 4	gi 30264669 ref NP_847046.	-	-3.875	4,381,656.61
73	S-layer protein EA1	gi 30261021 ref NP_843398.	-	-1.841	2,468,429.04
74	S-layer protein EA1 1	gi 30261021 ref NP_843398.	-	-1.445	3,879,124.54
75	S-layer protein EA1 2	gi 30261021 ref NP_843398.	-	-1.732	6,608,439.65
76	S-layer protein EA1 4	gi 30261021 ref NP_843398.	-83.307	-1.326	10,915,759.86
77	S-layer protein EA1 5	gi 30261021 ref NP_843398.	-36.429	-1.197	15,882,586.65
78	S-layer protein EA1 6	gi 30261021 ref NP_843398.	-28.081	-1.177	21,879,514.66
79	S-layer protein EA1 7	gi 30261021 ref NP_843398.	-29.970	-1.130	23,253,696.26
80	S-layer protein EA1 8	gi 30261021 ref NP_843398.	-	-2.685	1,812,403.16
81	S-layer protein EA1 9	gi 30261021 ref NP_843398.	-	1.654	551,725.25
82	S-layer protein Sap	gi 30261020 ref NP_843397.	-	-1.882	15,536,360.18
83	S-layer protein Sap 1	gi 30261020 ref NP_843397.	-	-1.660	23,058,130.30
84	S-layer protein Sap 2	gi 30261020 ref NP_843397.	-	-1.548	32,165,952.88
85	S-layer protein Sap 3	gi 30261020 ref NP_843397.	-83.240	-1.426	36,225,777.31
86	S-layer protein Sap 4	gi 30261020 ref NP_843397.	-	-2.113	30,448,394.96
87	S-layer protein Sap 6	gi 30261020 ref NP_843397.	-	-2.798	2,969,921.99
88	S-layer protein, putative	gi 30263247 ref NP_845624.	-	-1.185	3,042,874.10
89	S-layer protein, putative 1	gi 30263247 ref NP_845624.	-	-2.905	2,398,134.55
90	S-layer protein, putative 2	gi 30263247 ref NP_845624.	-	-5.376	2,376,199.19
91	oligopeptide ABC transporter, oligopeptide-binding protein, putative	gi 30260381 ref NP_842758.1	-	-2.750	1,371,914.53
92	oligopeptide ABC transporter, oligopeptide-binding protein, putative	gi 30260381 ref NP_842758.1	-	-10.698	1,595,516.96
93	S-layer protein EA1 10	gi 30261021 ref NP_843398.	-	-1.985	2,367,199.10
94	spermidine synthase	gi 30265393 ref NP_847770.	-	-4.280	2,884,061.16
95	sulfatase	gi 30265259 ref NP_847636.	-2.964	-1.290	3,508,783.62
96	sulfatase 1	gi 30265259 ref NP_847636.	-4.011	-1.129	12,905,031.07
97	sulfatase 2	gi 30265259 ref NP_847636.	-20.429	-1.968	5,488,730.46
98	sulfatase 3	gi 30265259 ref NP_847636.	-	-1.316	1,240,492.98
99	sulfatase 4	gi 30265259 ref NP_847636.	-17.441	-1.196	5,782,537.75
100	sulfatase 5	gi 30265259 ref NP_847636.	-	-1.138	3,502,366.92
101	superoxide dismutase, Mn	gi 30264347 ref NP_846724.	-	-3.791	9,392,367.20

102 superoxide dismutase, Mn 1	gi 30264347 ref NP_846724.	-	-2.426	15,662,433.01
103 tellerium resistance protein	gi 30260567 ref NP_842944.	-	-6.319	2,849,288.17
104 tellerium resistance protein 1	gi 30260567 ref NP_842944.	-7.536	-3.027	5,884,973.08
105 thioredoxin	gi 30264587 ref NP_846964.	-	-	2,306,260.95
106 transketolase	gi 30263628 ref NP_846005.	-	-3.223	6,289,159.48
107 transketolase 1	gi 30263628 ref NP_846005.	-	-4.055	3,007,382.89
108 transketolase 2	gi 30263628 ref NP_846005.	-	-6.272	1,401,021.41
109 translation elongation factor G	gi 30260298 ref NP_842675.	-7.031	-3.104	1,838,678.50
110 translation elongation factor G 1	gi 30260298 ref NP_842675.	-	-2.476	1,411,608.86
111 triosephosphate isomerase	gi 30265163 ref NP_847540.	-	-1.579	3,600,353.08
112 triosephosphate isomerase 1	gi 30265163 ref NP_847540.	-	-1.210	6,219,344.39
113 zinc-binding lipoprotein adca domain protein, (pxo1-130)	gi 47566506 ref YP_022458.1	-	-3.143	5,185,226.87
114 zinc-binding lipoprotein adca domain protein, (pxo1-130) 1	gi 47566506 ref YP_022458.1	-	-3.357	12,403,568.20
115 zinc-binding lipoprotein adca domain protein, (pxo1-130) 2	gi 47566506 ref YP_022458.1	-	-	6,323,453.54
116 zinc-binding lipoprotein adca domain protein, (pxo1-130) 3	gi 47566506 ref YP_022458.1	-	-11.521	5,399,576.11
117 zinc-binding lipoprotein adca domain protein, (pxo1-130) 4	gi 47566506 ref YP_022458.1	-	-	1,604,999.96
118 zinc-binding lipoprotein adca domain protein, (pxo1-130) 5	gi 47566506 ref YP_022458.1	-	-	3,691,019.16
119 zinc-binding lipoprotein adca domain protein, (pxo1-130) 6	gi 47566506 ref YP_022458.1	-	-2.615	4,364,397.10

Table 5 Presence of *B. anthracis* RA3R proteins in the different subproteomes

gi	name	Membranes	Cytosol	Secretome
gi 47566388	hypothetical protein GBAA_pXO1_0067 [Bacillus anthracis str. 'Ames Ancestor'].	x		
gi 47525266	pyridoxine biosynthesis protein	x	x	x
gi 47525303	ribose-phosphate pyrophosphokinase	x	x	
gi 47525317	hypoxanthine phosphoribosyltransferase		x	
gi 47525321	cysteine synthase A	x	x	x
gi 47525354	ribosomal protein L10	x	x	
gi 47525355	ribosomal protein L7/L12		x	
gi 47525363	translation elongation factor G	x	x	x
gi 47525364	translation elongation factor Tu	x	x	
gi 47525393	dna-directed rna polymerase, alpha subunit		x	
gi 47525412	arginase		x	
gi 47525416	glucosamine--fructose-6-phosphate aminotransferase (isomerizing)		x	
gi 47525525	chaperonin, 10 kda		x	x
gi 47525527	chaperonin, 60 kDa		x	x
gi 47525554	phosphoribosylaminoimidazole-succinocarboxamide synthase	x	x	
gi 47525557	phosphoribosylformylglycinamide synthase ii		x	
gi 47525561	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase		x	x
gi 47525562	phosphoribosylamine--glycine ligase		x	
gi 47525572	delta-1-pyrroline-5-carboxylate dehydrogenase, putative	x	x	
gi 47525588	glutamyl-tRNA(Gln) amidotransferase, B subunit		x	
gi 47525611	alkyl hydroperoxide reductase, F subunit		x	
gi 47525612	alkyl hydroperoxide reductase, subunit C	x	x	x
gi 47525619	pyridine nucleotide-disulfide oxidoreductase family protein	x		
gi 47525671	tellurium resistance protein		x	x
gi 47525779	formate acetyltransferase		x	
gi 47525869	alanine dehydrogenase	x		
gi 47525898	8-amino-7-oxononanoate synthase, putative		x	
gi 47525977	hypothetical protein GBAA0696			x
gi 47526138	glyceraldehyde-3-phosphate dehydrogenase, nadp-dependent		x	
gi 47526172	S-layer protein Sap			x
gi 47526173	S-layer protein EA1	x	x	x
gi 47526398	s-layer protein, putative	x		
gi 47526402	isocitrate lyase	x	x	x
gi 47526421	ornithine aminotransferase		x	
gi 47526447	3-oxoacyl-(acyl-carrier-protein) synthase iii		x	
gi 47526449	3-oxoacyl-(acyl-carrier-protein) synthase ii	x	x	
gi 47526458	oligopeptide abc transporter, atp-binding protein	x	x	
gi 47526461	oligopeptide ABC transporter, oligopeptide-binding protein			x
gi 47526497	enoyl-(acyl-carrier-protein) reductase	x		
gi 47526592	phap protein		x	
gi 47526596	3-oxoacyl-(acyl-carrier-protein) reductase, putative	x	x	
gi 47526625	oligoendopeptidase f, putative		x	
gi 47526793	ribosomal protein s1		x	
gi 47526810	nucleoside diphosphate kinase, putative	x	x	x
gi 47526862	thermostable carboxypeptidase 1		x	
gi 47526874	rhodanese-like domain protein		x	
gi 47527059	fumarate hydratase, class II		x	x
gi 47527281	conserved hypothetical protein		x	
gi 47527311	general stress protein			x
gi 47527328	nadh:flavin oxidoreductase / nadh oxidase family protein		x	
gi 47527399	formate--tetrahydrofolate ligase		x	
gi 47527483	conserved hypothetical protein		x	
gi 47527531	conserved hypothetical protein		x	x
gi 47527536	conserved hypothetical protein		x	
gi 47527564	alcohol dehydrogenase, zinc-containing	x	x	
gi 47527607	sporulation-control protein Spo0M, putative		x	
gi 47527616	mrr restriction protein-related		x	
gi 47527663	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	x		
gi 47527664	isochorismate synthase Dhbc	x	x	
gi 47527665	2,3-dihydroxybenzoate-amp ligase	x	x	
gi 47527666	isochorismatase	x	x	
gi 47527785	aminoacyl-histidine dipeptidase		x	
gi 47527837	acetyl-coa carboxylase, biotin carboxylase, putative	x	x	
gi 47528119	inorganic pyrophosphatase, manganese-dependent		x	
gi 47528252	chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase		x	
gi 47528623	S-layer protein, putative	x		x
gi 47528756	acetyltransferase, gnat family		x	
gi 47528880	cold shock protein CspB			x
gi 47528895	aldehyde dehydrogenase	x	x	
gi 47528961	aconitate hydratase 1		x	
gi 47529026	N-acetylmuramoyl-L-alanine amidase, family 2	x		x

gi 47529034	transketolase	x	x	x
gi 47529226	dihydrodipicolinate synthase		x	
gi 47529228	aspartate-semialdehyde dehydrogenase		x	
gi 47529234	polyribonucleotide nucleotidyltransferase			x
gi 47529252	ribosome recycling factor		x	
gi 47529254	translation elongation factor Ts		x	
gi 47529256	transcriptional regulator CodY		x	
gi 47529263	succinyl-CoA synthase, alpha subunit	x	x	
gi 47529281	malonyl CoA-acyl carrier protein transacylase	x		
gi 47529477	pyruvate dehydrogenase complex e3 component, dihydrolipoamide dehydrogenase	x		
gi 47529479	pyruvate dehydrogenase complex e1 component, beta subunit	x	x	
gi 47529480	pyruvate dehydrogenase complex e1 component, alpha subunit		x	
gi 47529533	acetyl-CoA acetyltransferase		x	
gi 47529560	phosphoenolpyruvate-protein phosphotransferase		x	
gi 47529561	phosphocarrier protein hpr			x
gi 47529575	peptidyl-prolyl cis-trans isomerase, cyclophilin-type		x	
gi 47529678	3-methyl-2-oxobutanoate dehydrogenase, beta subunit		x	
gi 47529682	leucine dehydrogenase	x	x	x
gi 47529715	proline dipeptidase		x	x
gi 47529794	superoxide dismutase, Mn		x	x
gi 47529836	chaperone protein dnaK	x	x	
gi 47529904	transcription elongation factor grea		x	
gi 47529967	abc transporter, atp-binding protein	x		
gi 47529983	rod shape-determining protein mreB	x	x	
gi 47530004	trigger factor		x	
gi 47530053	succinate dehydrogenase, flavoprotein subunit		x	
gi 47530057	thioredoxin		x	x
gi 47530058	electron transfer flavoprotein, alpha subunit	x	x	
gi 47530059	electron transfer flavoprotein, beta subunit		x	
gi 47530112	peptidase, m42 family			x
gi 47530132	malate dehydrogenase	x	x	
gi 47530138	pyruvate kinase	x	x	x
gi 47530166	alanine dehydrogenase	x	x	x
gi 47530183	acetate kinase	x	x	
gi 47530223	catabolite control protein A			x
gi 47530293	leucyl-trna synthetase		x	
gi 47530320	s-adenosylmethionine synthetase	x		
gi 47530362	hypothetical protein GBAA5061		x	
gi 47530411	naphthoate synthase		x	
gi 47530429	L-lactate dehydrogenase		x	
gi 47530435	phosphoglucose isomerase		x	x
gi 47530456	UTP-glucose-1-phosphate uridylyltransferase	x		
gi 47530458	phosphoglucomutase/phosphomannomutase family protein		x	
gi 47530465	pyridine nucleotide-disulphide oxidoreductase		x	
gi 47530526	abc transporter, substrate-binding protein, putative		x	
gi 47530551	acyl-CoA dehydrogenase		x	
gi 47530553	acetyl-coa acetyltransferase		x	
gi 47530597	general stress protein 20U	x	x	x
gi 47530623	tyrosyl-trna synthetase	x		
gi 47530674	enolase	x	x	x
gi 47530675	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	x	x	x
gi 47530678	phosphoglycerate kinase		x	
gi 47530679	glyceraldehyde 3-phosphate dehydrogenase		x	
gi 47530690	atp-dependent clp protease, proteolytic subunit clpp		x	
gi 47530729	cell division abc transporter, atp-binding protein ftse		x	
gi 47530746	udp-n-acetylglucosamine 2-epimerase		x	
gi 47530784	sulfatase			x
gi 47530838	mbi protein	x		
gi 47530867	ATP synthase F1, beta subunit	x	x	
gi 47530869	ATP synthase F1, alpha subunit		x	
gi 47530871	atp synthase f0, b subunit	x		
gi 47530878	serine hydroxymethyltransferase	x	x	
gi 47530901	fructose-bisphosphate aldolase, class II	x	x	
gi 47530963	conserved hypothetical protein			x
gi 47531060	ribosomal protein S6		x	
gi 47566388	hypothetical protein GBAA_pXO1_0067		x	
gi 47566400	surface layer protein, (pxo1-54)	x		
gi 47566444	S-layer protein, (pxo1-90)	x		
gi 47566476	protective antigen			x
gi 47566484	lethal factor			x
gi 47566506	zinc-binding lipoprotein adca domain protein, (pxo1-130)	x	x	
gi 47777770	stage V sporulation protein G		x	
gi 47777798	GMP synthase		x	
gi 47777948	3-methyl-2-oxobutanoate hydroxymethyltransferase	x		

gi 47778099	3-phosphoshikimate 1-carboxyvinyltransferase	x		
gi 47778224	reca protein, group i intron-containing	x		
gi 47778312	mta/sah nucleosidase		x	
gi 47778335	isocitrate dehydrogenase, NADP-dependent	x	x	
gi 47778339	thiol peroxidase		x	
gi 47778387	iron compound ABC transporter, iron compound-binding protein		x	
gi 47778395	triosephosphate isomerase	x	x	x
gi 47778412	fructose-1,6-bisphosphatase, class II	x	x	
gi 50196912	glutamyl-tRNA(gln) amidotransferase, a subunit		x	
gi 50196927	aspartate aminotransferase		x	
gi 50196970	spermidine synthase			x